J. Exerc. Nutr. Biochem. 2015;19(2):107-113
ISSN : 2233-6834 (Print) ISSN : 2233-6842 (Online)
http://dx.doi.org/10.5717/jenb.2015.15052508

JENB
Exercise Nutrition & Biochemistry

ORIGINAL ARTICLE

Change of gene expression on protein uptake composition and hindlimb-suspension in rat skeletal muscle

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(History: Received: 2015/05/07, Revised: 2015/05/20, Accepted: 2015/05/25, Published: 2015/06/30)

Wookwang Cheon and Kiwon Lim. Change of gene expression on protein uptake composition and hindlimb-suspension in rat skeletal muscle. *JENB*., Vol. 19, No. 2, pp.107-113, 2015 **[Purpose]** This study was to investigate changes in BCAT and BCKDH genes by Hindlimb-Suspension (HS) and protein intake composition (casein, Whey protein) in rats. **[Methods]** Following 5-day preliminary feeding, forty-eight male 5 weeks old Sprague Dawley albino rats (110g) divided into 17% protein intake group (24 rats) and 30% protein intake group (24 rats), and each group divided further into Hindlimb-Suspension group (HS; 12 rats) and control group(CON; 12 rats). Eventually, this study was performed with Whey protein intake group (HS; 6 rats, CON; 6 rats) and casein intake group (HS; 6 rats, CON; 6 rats). For analysis purposes, total RNA was extracted from isolated skeletal muscles, and mRNA expression was analyzed using Real Time PCR. Two-way ANOVA was performed to examine the difference in BCATm and BCKDH mRNA expression on protein uptake and myoatrophy. post-hoc test was perform on interaction if any, and significance level was set at $p < 0.05$. **[Results]** In this study, BCATm and BCKDH gene analysis in rat skeletal muscles by hindlimb-suspension and protein intake composition resulted in significant higher BCATm expression in 30% dietary protein group and hindlimb-suspension group that control group. In addition, regarding BCKDH, BCKDH was significantly higher in hindlimb-suspended 30% protein intake group than control group. **[Conclusion]** Overall, protein intake and myoatrophy demonstrated close relationship in skeletal muscles. Therefore, it is likely to affect effectively in prevention or recovery of exercise induced muscle disorder. This effect is considered to be applied to maintain and improve health of not only athletes but also the general public. Additionally it would be applied in convalescent rehabilitation due to skeletal muscle atrophy. **[Key words]** Rat Skeletal muscle, Whey protein, BCAT, BCKDH, Real-Time PCR

INTRODUCTION

Myoatrophy of skeletal muscle is induced by muscular inactivity, aging etc., thus for athletes and the aged, it is important to increase and maintain skeletal muscle mass. Protein intake is important to maintain muscle protein, and the effect probably depends on the amino acid composition in protein intake. Milk protein is composed of casein and Whey protein, and Whey protein shows more rapid digestion and absorption than casein, and contains more branched-chain amino acids (BCAA) which promote protein synthesis than casein.

Amino acid makes up protein, and is an important component of human body. Nine of twenty proteinogenic amino acids are called "essential" for humans and cannot be created from other compounds by the human body. Branched- Chain Amino Acids (BCAA) are amino acids having aliphatiac side-chains with a branch, and valine, leucine, and isoleucine are included. Because these 3 amino acids are essential and so, BCAA must be taken in as food.

Because a BCAA is not synthesized in the body, only the catabolic system is equipped in the human and animal cell. Each catabolic system for BCAAs [1], which is located in mitochondria contains many steps, and ultimately synthesizes precursors or intermediates of TCA cycle. Also, initial two catabolic reactions are common to the all of three BCAAs, and characterize the BCAA catabolic system [2].

First step of the BCAA catabolism is a reversible reaction

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catalyzed by BCAT(branched-chain aminotransferase) and BCAT reaction is triggered by substantial intracelluar accumulation of BCAA. This is for the purpose of preferential utilization of BCAA in protein synthesis. Recent study has shown that there are two BCAT isoenzymes, one located in mitochondria (BCATm) and the other in the cytosol (BCATc) [3].

Second reaction is catalyzed by BCKDH(branched-chain-keto acid dehydrogenase) complex. This enzyme reaction is oxidative decarboxylation and irreversible, therefore it is considered that this step regulates all BCAA catabolism [2].

Interorgan difference of BCAA metabolism has been reported. This is resulted from substantial interorgan difference of enzyme activity in the initial two steps, that is, in skeletal muscles BCAT activity is very high, whereas BCKDH activity is restricted. On the contrary, it is reported that in liver, BCAT is almost inactive, whereas BCKDH complex is highly active [4].

Skeletal muscle is the largest tissue and accounts for 40% of body weight in human body. BCAAs account for approximately 35% of essential amino acids composing of skeletal muscle protein, furthermore it is reported that BCAAs account for approximately 50% of essential amino acids in food (approximately 20% of total amino acids) [2]. Therefore, plenty of BCAA is accumulated in human body, and taken by meals.

Free BCAAs in human body is known as an ingredient in protein synthesis and a regulator in protein metabolism concurrently. BCAAs, especially leucine have functions to promote protein synthesis and inhibit the catabolism. It is known that leucine stimulates protein synthesis by mRNA translation. Additionally, it is reported that effect of leucine on protein anabolism is possibly demonstrated by stimulating insulin release [1,2].

In the study on timing of post-exercise protein or amino acid intake for muscle protein synthesis, it is reported that immediate intake of protein supplement after exercise was more effective than time-divided intake after exercise [5,6], while immediate intake of essential amino acid mixture supplement before exercise was more effective than immediate intake after exercise [7]. It is demonstrated that muscle protein catabolism is promoted by exercise, in healthy male adults oral BCAA supplementation at 45 min and 25 min before exercise results in BCAA concentration increase in arterial blood and muscle [8], and amino acid supplementation may substantially suppress plasma CK activity induced by high intensity exercise [9,10]. Also, it has been reported that in untrained sedentary individuals BCAA supplementation before unaccustomed squat exercise may reduce muscle damage such as myalgia and muscle fatigue [11].

BACC content of milk protein is approximately 22%, which is relatively high compared to other animal or vegetable protein (BCAA content: 15~20%). Milk protein consists of about 80% of casein and about 20% of Whey protein, and Whey protein shows higher BCAA content; 20% and 26% in casein and Whey protein, respectively [12]. Furthermore, because Whey protein is readily digested compared to casein, it has been reported that peak plasma leucine concentration is reached at approximately 1 hour post-dose [13,14].

BCAA is likely to affect effectively in prevention or recovery of exercise induced muscle disorder. This effect is considered to be applied to maintain and improve health of not only athletes but also the general public. Additionally it would be applied in convalescent rehabilitation due to skeletal muscle atrophy.

In vivo BCAA activation by Whey protein intake is anticipated to promote muscle synthesis and suppress myoatrophy effectively. Also, it may be important to evaluate changes in BCAT and BCKDH genes considered as critical factors on functions and expressions of various BCAA related elements. Therefore, this study is to investigate changes in BCAT and BCKDH genes by protein intake levels (17% vs 30%) and Whey protein intake and Hindlimb-Suspension (HS) in rats.

METHODS

Subjects

Forty-eight male 5 weeks old Sprague Dawley albino rats (110g) were purchased (SLC, Shizuoka, Japan) and individually houses in wire cages and maintained on a 12 hour light/dark cycle (light 20:00~08:00, dark 08:00~20:00) at constant room temperature 22 ± 2℃ and relative humidity 60%. During 5 days preliminary feeding, the diets were based on the AIN-93G(Kurea, Tokyo, Japan) diet with water. During 6 days dietary intake, protein contents was 17.08% (17% protein intake group) and 30% (30% protein intake level 30% group) base on the AIN-93G in both dietary casein and Whey protein group. Casein diet was based on the AIN-93G diet, and Whey protein diet was based on the AIN-93G with casein substituted by Whey protein.

Study design

Following 5-day preliminary feeding, forty-eight male 5 weeks old Sprague Dawley albino rats (110g) divided into

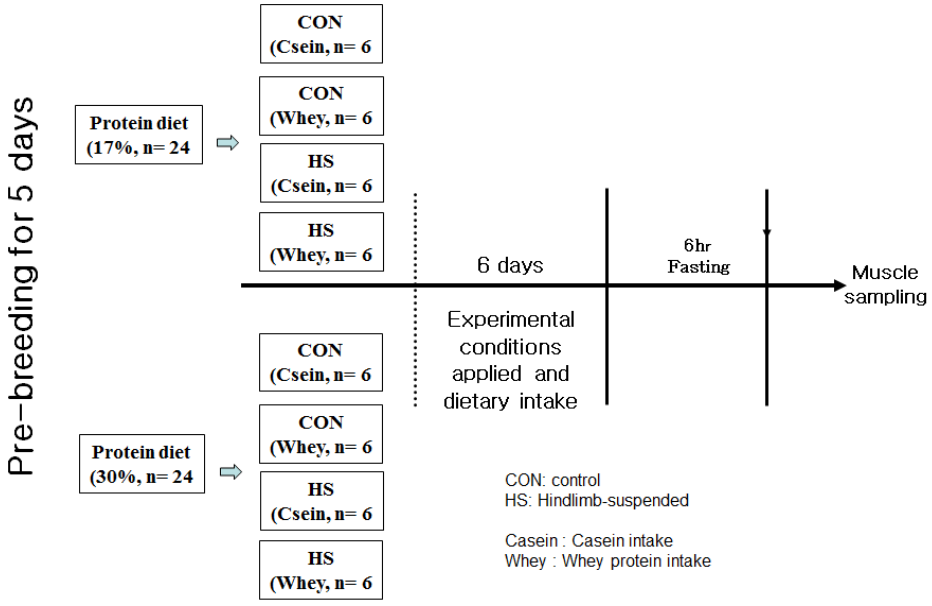

Fig. 1. Experiment design

17% protein intake group (24 rats) and 30% protein intake group (24 rats), and each group divided further into Hindlimb-Suspension group (HS; 12 rats) and control group(CON; 12 rats). Eventually, this study was performed with Whey protein intake group (6 rats) and casein intake group (6 rats) according to intake level and hindlimb-suspension <Fig. 1>.

For dietary composition, during 5 days preliminary feeding, the diets were based on the AIN-93G(Kurea, Tokyo, Japan) diet with water. During 6 days dietary intake, protein contents was 17.08%(17% protein intake group)와 30%(30% protein intake level 30% group) base on the AIN-93G in both dietary casein and Whey protein group. Casein diet was based on the AIN-93G diet, and Whey protein diet was based on the AIN-93G with casein substituted by Whey protein.

Analysis items and methods

Skeletal muscle isolation and total RNA concentration analysis

Gastrocnemius muscles were collected from rats, weighed and stored with RNAlater(QIAGEN, Tokyo, Japan) containing RNase inhibitor at -70℃ for use in extraction of total RNA. For total RNA extraction, 30-40mg of skeletal muscle was homogenized and RNA was extracted from homogenates using kit (RNeasy Fibrous Tissue Midi Kit, QIAGEN, Tokyo, Japan). Extracted RNA was measured spectrophotometrically at 260nm, and prior to experiment, rRNA 18S and rRNA 28S bands were confirmed based on the electrophoresis with 0.5ug of RNA in 1% agarose gel.

mRNA expression analysis using Real Time PCR

For real time PCR, primer was prepared using primer3 version 0.4(Web software provided by Steve Rozen and Whitehead Institute for Biomedical Research.) etc. Primers used were sense and anti-sense primers on UORF (upstream open reading frame) or ORF of gene sequence.

Total RNA expression level after exercise and during convalescence quantified and analyzed using iCycler iQ Multicoolor Real-Time PCR Detection System (Bio-Rad

Table 1. Component of Kit

Reagent	Concentration/reaction)
2× One Step SYBR RT-PCR buffer	10 ul
TaKARa Ex Taq HS (5 U/ul)	0.4 ul
M-MLVRTase (RNaseH free) (200 U/ul)	0.2 ul
RNase Ingibitor (40 U/ul)	0.4 ul
PCR Forward Primer (10 U/ul)	0.4 ul
PCR Reverse Primer (10 U/ul)	0.4 ul
total RNA	2 ul
RNase Free dH2O	6.2 ul
Total	20ul

Table 2. Protocol of Real Time PCR

Step	Reps	Temperature (℃)	Time(s)
Reverse Transcription	1	45	900
		95	120
PCR Reaction	40	95	10
		60	45
		72	30
Melting Curve	46	72	10

Table 3. Primer sequence

Gene		Primer sequence
GAPDH	Sense	5'-GCCCAGCAATACTGAGA-3'
	Anti-Sense	5'-GGGTGCAGCGAACTTTATTG-3'
BCAT2		5'-AGGGAAGGTCGTTCCAGACT-3'
		5'-GTCCAAATTACATGGGTCCG-3'
BCKDH		5'-CCATCTCCACACCAACCTCT-3'
		5'-TACACCGCAAACACATCGTT-3'

Laboratiries, Inc, USA). Pre-defined mRNA level in preliminary experiment using standards was used. One Step SYBR RT-PCR kit(TaKaRa, JAPAN) was used, and specific components of reagent and protocol of real time PCR are presented in <Table 1> and <Table 2>, respectively.

Data management and statistic methods

For statistics, means and standard deviations of all items were calculated using SPSS 21.0 statistical package, two-way ANOVA was performed to examine the difference in BCATm and BCKDH mRNA expression on protein uptake and hindlimb-suspension. Post-hoc test was perform on interaction if any, and significance level was set at $p < 0.05$.

RESULTS

This study was to investigate changes in BCAT and BCKDH genes by protein intake levels (17% vs 30%) and Whey protein intake and Hindlimb-Suspension (HS) in rats and the results are as follows.

BCATm gene analysis

As shown in <Table 4>, in the change in the expression level of BCAT2 mRNA by 6 days protein intake level groups (whey protein intake) and hindlimb-suspension (HS), main effect of protein intake level [$F(1, 88)] = 28.536$, $p < .001$] was significant, and main effect of group [$F(3, 88)] = 22.140$, $p < .001$] was also significant. In addition, interaction effect between protein intake level and group [$F(3, 88)] = 6.401$, $p < .001$] was detected significantly. Therefore, ad-hoc test on the interaction demonstrated that BCATm expression was significantly higher in the HS Whey protein group than CON casein group in the 17% protein intake group ($p < .05$), and significantly higher in the HS casein group and whey protein group compared to CON casein group and Whey protein group, respectively($p < .05$) in the 30% protein intake group. For hindlimb-suspension, BCATm expression levels in HS casein group and Whey protein group were significantly higher in the 30% protein intake group than 17% protein intake group ($p < .05$).

As shown in <Table 5>, in the change in the expression level of BCATm/GAPDH mRNA by 6 days protein intake level groups (whey protein intake) and hindlimb-suspension

Table 4. The change of expression gene BCATm mRNA on Hindlimb and intake protein 17%, 30% in Rat skeletal muscle

Group Protein (%)	Control		Hindlimb-Suspended		F
	Casein	Whey	Casein	Whey	
17% Protein	22.56 ± 3.35[a]	24.97 ± 6.63	26.74 ± 6.52	29.47 ± 5.49[d]	3.407* a < d
30% Protein	22.06 ± 5.13[a]	28.34 ± 2.70[b]	39.71 ± 10.33[c###]	39.81 ± 6.01[d###]	21.990*** a,b < c,d
Protein (%)			28.536***		
Group F			22140***		
Protein×Group			6.401***		

Values : Mean ± SD *$p < .05$, ***$p < .001$,
###$p < .001$ Significant difference from 17% Protein

Table 5. The change of expression gene BCATm/GAPDH mRNA on Hindlimb and intake protein 17%, 30% in Rat skeletal muscle

Group Protein (%)	Control		Hindlimb-Suspended		F
	Casein	Whey	Casein	Whey	
17% Protein	0.90 ± 0.12	1.01 ± 0.32	0.98 ± 0.20	1.10 ± 0.29	1.376
30% Protein	0.92 ± 0.17[a]	0.96 ± 0.16	1.18 ± 0.40	1.28 ± 0.48[d]	3.391* a < d
Protein (%)			2.255		
Group F			4.296**		
Protein×Group			1.053		

Values : Mean ± SD *$p < .05$, ***$p < .001$

Table 6. The change of expression gene BCKDH mRNA on Hindlimb and intake protein 17%, 30% in Rat skeletal muscle

Group Protein (%)	Control		Hindlimb-Suspended		F
	Casein	Whey	Casein	Whey	
17% Protein	26.18 ± 2.00	27.89 ± 3.15[b]	23.85 ± 4.79[c]	25.90 ± 3.05	2.857* b>c
30% Protein	26.70 ± 2.70	31.43 ± 5.79	32.80 ± 8.50[###]	32.31 ± 4.40[###]	2.834*
Protein (%)			25.376***		
Group F			2.122		
Protein×Group			3.557*		

Values : Mean ± SD *p < .05, ***p < .001,
[###]p < .001 Significant difference from 17% Protein

Table 7. The change of expression gene BCKDH/GAPDH mRNA on Hindlimb and intake protein 17%, 30% in Rat skeletal muscle

Group Protein (%)	Control		Hindlimb-Suspended		F
	Casein	Whey	Casein	Whey	
17% Protein	1.04 ± 0.06	1.12 ± 0.21[b]	0.88 ± 0.17[c]	0.95 ± 0.17	4.937** b>c
30% Protein	1.13 ± 0.18	1.05 ± 0.17	0.97 ± 0.34	0.98 ± 0.23	0.377
Protein (%)			0.684		
Group F			3.699*		
Protein×Group			0.824		

Values : Mean ± SD *p < .05, **p < .01

(HS), main effect of protein intake level was not significant, while main effect of group [F(3, 88)] = 4.296, p < .01]was significant. However, interaction effect between protein intake level and group was not significant. Ad-hoc test on the main effect of group demonstrated that BCATm expression was significantly higher in the HS Whey protein group compared to CON casein group (p < .05) in the 30% protein intake group.

BCKDH gene analysis

As shown in <Table 6>, in the change in the expression level of BCKDH mRNA by 6 days protein intake level groups (whey protein intake) and hindlimb-suspension (HS), main effect of protein intake level F(1, 88)] = 25.376, p < .001]was significant, while main effect of group was not significant. However, interaction effect between protein intake level and group [F(3, 88)] = 3.557, p < .05] was detected significantly. Ad-hoc test on the interaction demonstrated that BCKDH mRNA expression was significantly lower in the HS casein group than CON Whey protein group in the 17% protein intake group (p < .05), while there was not significant difference in the 30% protein intake group. For hindlimb-suspension, BCKDH mRNA levels in HS casein group and Whey protein group were significantly higher in the 30% protein intake group than 17% protein intake group (p < .05).

As shown in <Table 4>, in the change in the expression level of BCKDH/GAPDH mRNA by 6 days protein intake level groups (whey protein intake) and hindlimb-suspension (HS), main effect of protein intake level was not significant, while main effect of group [F(3, 88)] = 3.699, p < .05]was significant. However, interaction effect between protein intake level and group was not significant. Ad-hoc test on the main effect of group demonstrated that BCKDH expression was significantly higher in the HS Whey protein group compared to CON casein group (p < .05) in the 17% protein intake group.

DISCUSSION

As shown in the result of this study, in both 17% and 30% protein intake group, BCAT2 gene expression was higher in HS and Whey protein intake group.

Previous studies reported that, dietary intervention with vitamin D, calcium and Whey protein increased lean mass, and reduced fat mass in rats [15], and that in 117 obese male adults and 210 obese female adults with metabolic syndrome, whey protein twice daily in conjunction with resistance (2 d/wk) and aerobic (1 d/wk) exercise training didn't affect body weight, but increased lean body mass, and decreased fat mass [16]. In addition, Whey proteins was reported to be more efficient than casein in the recovery of muscle functional properties in rats [17].

For hindlimb-suspension, it has been reported that 15 days hindlimb-suspension in rats reduced significantly cross-sectional area of skeletal muscle [18], and that oral BCAA supplementation in hindlimb-suspended rats inhibited Atrogin-1 and MuRF1 involved in skeletal muscle catabolism and prevented myoatrophy [19]

In human skeletal muscle BCAT activity is high, so BCAAs are important donor of amino groups. For example, in skeletal muscle, amino groups are transferred to pyruvic acid and alanine is synthesized. The alanine is transported to liver by blood circulation and used for gluconeogensis [20]. As shown in this study results, it is considered that high BCAT2 activity promotes BCAA degradation following Whey protein administration along with skeletal muscle reduction due to hindlimb suspension and may help prevent skeletal muscle atrophy. However, to date BCAA metabolism on hindlimb-suspension and Whey protein administration has not yet been elucidated.

In the BCKDH gene analysis result, only hindlimb suspended 30% protein intake group showed higher BCKDH gene expression than 17% protein intake group. Low BCKDH complex activity in skeletal muscle is attributed to high tissue kinase expression, and more bound form of kinase [21].

Human skeletal muscle is considered as the major tissue of BCAA metabolism. This is attributed to large amount of skeletal muscle relative to whole body and higher BCAT activity in skeletal muscle than other organs. It is reported that this activity is enhanced by exercise training [5]. Several authors investigated effect of exercise on BCAT activity in rat skeletal muscle, however there was no significant effect. Because the activity in skeletal muscle is high basically, the meaning of effect of training is considered small.

In other tissues beside liver, especially muscle, BCAA catabolism is regulated by BCKDH complex. Even in liver, this enzyme complex and thereafter catabolic system exist, therefore liver BCKDH complex functions in the catabolism of branched chain-keto acid formed by transamination. Especially in rat liver, the enzymatic activity is substantially high, therefore the role of BCKDH complex in liver may be major [22].

To date, accurate BCAA dosage for human has not yet been determine, however regarding myoatrophy the dosage will be increased gradually.

As described above, BCAA catabolism is largely affected by myoatrophy and protein intake and regulated by various hormones related to protein synthesis and gluconegenesis. Detailed mechanism is the subject for future research, and BCAA is believed to be the amino acid closely related to energy metabolism and protein metabolism.

CONCLUSIONS

The conclusion was derived from changes in BCAT2 and BCKDH mRNA expression by protein intake levels, Hindlimb-Suspension (HS) and protein intake composition as follows.

Expressions of both BCAT2 and BCKDH genes were significantly higher in hindlimb-suspended 30% protein intake group. From this result, protein intake and myoatrophy demonstrated close relationship in skeletal muscles. Therefore, it is likely to affect effectively in prevention or recovery of exercise induced muscle disorder. This effect is considered to be applied to maintain and improve health of not only athletes but also the general public. Additionally it would be applied in convalescent rehabilitation due to skeletal muscle atrophy.

ACKNOWLEDGMENTS

This research was supported by 2008 Korea Research Foundation Grant, as part of new research grant by Korea Research Foundation (KRF-2008-0176-G00021).

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